Review

Biophysics of lipid bilayers containing oxidatively modified phospholipids: Insights from fluorescence and EPR experiments and from MD simulations ☆

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ABSTRACT

This review focuses on the influence of oxidized phosphatidylcholines (oxPCs) on the biophysical properties of model membranes and is limited to fluorescence, EPR, and MD studies. OxPCs are divided into two classes: A) hydroxy- or hydroperoxy-dieonyl phospatidylcholines, B) phospatidylcholines with oxidized and truncated *chains* with either aldehyde or carboxylic group. It was shown that the presence of the investigated oxPCs in phospholipid model membranes may have the following consequences: 1) decrease of the lipid order, 2) lowering of phase transition temperatures, 3) lateral expansion and thinning of the bilayer, 4) alterations of bilayer hydration profiles, 5) increased lipid mobility, 6) augmented flip-flop, 7) influence on the lateral phase organisation, and 8) promotion of water defects and, under extreme conditions (i.e. high concentrations of class B oxPCs), disintegration of the bilayer. The effects of class A oxPCs appear to be more moderate than those observed or predicted for class B. Many of the abovementioned findings are related to the ability of the oxidized chains of certain oxPCs to reorient toward the water phase. Some of the effects appear to be moderated by the presence of cholesterol. Although those biophysical alternations are found at oxPC concentrations higher than the total oxPC concentrations found under physiological conditions, certain organelles may reach such elevated oxPC concentrations locally. It is a challenge for the future to correlate the biophysics of oxidized phospholipids to metabolic studies in order to define the significance of the findings presented herein for pathophysiology. This article is part of a Special Issue entitled: Oxidized phospholipids—their properties and interactions with proteins.

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Contents

1. Introduction

Lipid oxidation elicits profound changes in the chemical structure of phospholipid fatty acids [1,2]. The polar and hydrophilic nature of the oxidized groups suggest a lower compatibility with the hydrophobic inner part of the bilayer [3] thus opening the possibility that typical properties such as fluidity, ordering, transition temperature, lateral organisation, polarity and permeability might be modified. If true, the altered bilayer properties might impact the functioning of integral proteins [4] whose optimal activity is often closely related and critically modulated by the bilayer's chemical and physical states [5]. Therefore, the detection and study of possible alterations in the bilayer properties are of high importance.

Following the proposal of the fluid mosaic model by Singer and Nicholson in 1972 [6] and its further development [7,8], the research focus remained for many decades on the characterization of bilayers composed of unaltered phospholipids and unmodified natural fatty acids. The impact of oxidized phospholipids (oxPLs) on the bilayer structure and properties was not systematically studied and, in fact, it is a common practice to use antioxidants (such as BHT and an argon or nitrogen experimental atmosphere) in order to prevent the formation of oxPL in samples. However, oxPLs are now strongly suspected of playing an important role in pathologies linked to oxidative stress induced by reactive oxygen species (ROS) [9] and are therefore a subject worthy of study.

From a historical point of view it is significant to note that the structure of cardiolipin (the most complex phospholipid that is still a simple organic molecule) was first established by van Deenen in 1966 [10], considerably later than the first announcement of DNA's much more intricate structure in 1953. This fact evidences the slow building of knowledge in the field of phospholipids which has led to a gap between lipidomics and genomics (and other -omics). Despite some early studies of the effects of oxidized lecithin on the bilayer asymmetry, phase behaviour and micelle formation [11], the gap kept on widening [12] also contributing to the overlooking of oxPLs and their chemical, physical and membrane properties. In the last decade there has been more focus on oxPLs due to research that pertains to their pathological and physiological roles in nature [9].

The concept of functional cooperativity [5] of the phospholipid bilayer is a relatively new one. The phospholipid bilayer takes on a physical identity on the basis of the individual phospholipid molecular species that build up its structure. The interplay between fatty acid residues, polar heads and cholesterol results in their enclosure in a manner particular to the membrane and to the membrane microdomains in which integral proteins are embedded. The resulting delicate architecture of the phospholipid–protein complex in a given membrane domain constitutes the basis for optimal functioning of the protein itself. Keeping in mind the critical role that the fatty acid residues play in fine tuning the chemical–physical properties of the bilayer, it is easy to imagine that a perturbation of the phospholipid ensemble surrounding a functional protein will perturb the protein's membrane integration and in consequence also its structure and functioning [4].

In pathologies related to oxidative stress, the phospholipid bilayer is perturbed by an increasing number of oxPL molecular species. These polar oxPLs conflict not only with the hydrophobic bilayer interior but also with non-polar amino acids in the hydrophobic part of proteins leading one to expect a possible impairment of the proteins' function. More dramatically, lipoperoxidation leading to carboxyacyl-phosphatidylcholines induces membrane disruption with straightforward pathological implications [13,14]. This reasoning constitutes the foundation for considering oxPL as a possible molecular base of oxidative stress-related pathologies at the membrane level. This view does not conflict with the pathological role of oxPL in signalling apoptosis. However, since this signalling does not involve the phospholipid bilayer as a whole, it will not be considered in the present review. Similarly, other specific pathological effects caused by oxPLs in contexts different from the phospholipid bilayer are also out of the scope of this review.

A variety of physical or chemical ROS initiators have been commonly used (UV- and X-rays, azo-compounds, t-Bu-hydroperoxide, Fe^{++}/H_2O_2, Cu^{++}) to give rise to lipid oxidation in model or natural membranes. The results are strongly dependent on both the nature of the membrane and the radical triggering conditions. Moreover, oxidation has been quantified mainly by malondialdehyde determination through the TBARS assay or by spectrophotometric detection of conjugated dienes at 235 nm wavelength, though both methods have significant problems. Malondialdehyde undergoes further oxidation to malonaldehyde [F.M.M., personal observation] which escapes determination by the TBARS assay thus leading to an underestimation of oxidation. Conjugated dienes represent only one of the oxidation cascade steps [14] and do not allow a determination of the following intermediate oxidized species and of the final molecules therefore also leading to underestimation. To prevent such drawbacks a reliable

strategy is to control the oxidation state of model membranes by mixing known amounts of pure and defined oxPL molecules with normal phospholipids, according to the approach used in earlier studies [15–17].

For this purpose classic oxidation protocols were eventually dropped and substituted with organic synthesis (or biosynthetic) methods in order to get pure oxidized phosphatidylcholine species instead of the inextricably high variety of oxidized molecules resulting from the use of oxidants [18]. Even so, these models do not necessarily reproduce natural oxidized membranes caused by exposure to physiologically or pathologically originated ROS. Nonetheless, it must be reasoned that not even a bilayer reconstituted from phospholipid extracts of natural membranes will reproduce the original membrane. The membrane's lipid topology is inevitably and irremediably modified in the lipid blend resulting from the very extraction step. Such lipid blend yields a reconstituted membrane having an overall average lipid composition different from any lipid microdomain present in the original natural membrane.

Notwithstanding, the characterization of model membranes containing oxPL will help understand the effects of the oxidized molecules on biological membranes, even without the knowledge as to the amount of oxidized species in a natural membrane. In fact, one must consider that natural membranes are characterised by the presence of lipid microdomains with different phospholipid compositions and unsaturation degrees. Following a ROS attack, fully unsaturated microdomains could well turn into fully oxidized microdomains while still representing a small percentage of the whole lipidome. Furthermore, defining the typical oxidation pattern of an oxidized natural membrane is still a difficult task. The pathways of oxidative stress, the variety of oxPLs formed (which depend on the oxidizing agent and the oxidation conditions) and the type of oxidative pathology are still to be fully understood. To this aim, mass spectrometry studies of oxidative lipidomics in pathological tissues have been undertaken [19,20]. In conclusion, while natural lipoperoxidized membrane composition cannot be precisely defined, the characterization of membrane models enriched in the species of interest can give first insights into the general effects of oxidative stress on the bilayer properties.

The increasing number of publications dealing with oxidation in well-defined model systems motivated the review of such contributions. Here are collected results of studies in which EPR, various fluorescence methods and computer simulations were used to elucidate the structure and physical properties of oxidized lipid bilayers. Other techniques have been applied to the study of lipid oxidation-dependent bilayer alteration, such as NMR and Differential Scanning Calorimetry [11,21], and X-ray diffraction [22], yet their use never became systematic nor generated standard methodologies for lipoperoxidaton studies. Moreover, the methods were applied to batch-oxidized membrane systems lacking any precise definition of the molecular structure of the oxPL involved and the present review aims at presenting updated knowledge on the cause–effect relationship existing between a given oxPL molecular structure and the consequently observed bilayer alteration. Some of the methods described herein were developed and first applied to batch-oxidized systems, and only later used for characterisation of model membranes with a well-defined composition. In those cases we mention the former contributions in Section 2 only to define the methods, while the results presented in the latter are discussed in detail in Section 3.

Interestingly, the lipoperoxidation studies are up to now limited mostly to oxidation products of the most abundant lipid class of phosphatidylcholines which we herein refer to as oxPC. Due to the limited scope of the present review, the reader interested in earlier works dealing with in-situ oxidation or experiments performed in vitro and in vivo is referred to the excellent review of Fruhwirth and co-authors [9].

2. EPR, fluorescence and MD applied to oxPL studies: introduction to the methods

2.1. EPR

Since the dynamic time scale of the inner phospholipid bilayer is of the same order of magnitude of conventional X-band electron paramagnetic resonance spectroscopy (EPR) time scale [23], the EPR spectrum of a magnetically anisotropic paramagnetic probe (the nitroxide) embedded into the bilayer (Fig. 1) is strongly affected by the fatty acid motional degree. Conversely, this dependence of EPR spectra is a telltale of the inner bilayer dynamics that, in turn, strongly depends on the type and structure of the fatty acids, so that also their physical and chemical modifications are reflected in the EPR spectrum line shape. As already noted, the polar nature of oxidized fatty acid residues hints at an incompatibility of oxygenated groups with the hydrophobic inner bilayer, thus suggesting that typical bilayer properties, hence the bilayer dynamics, may be altered in a way detectable by spectral variations of a paramagnetic probe EPR spectrum. Therefore, typical bilayer parameters and their variations related to the presence of oxPL can be monitored through the modifications of the spectrum of an EPR probe.

Hereafter only conventional X-band EPR applications will be described, though we would like to acknowledge that also W-band EPR (high frequency) [24], and electron spin echo envelope modulation (ESEEM) [25] can be useful in the study of local polarity and water penetration in oxPL bilayers in more detail.

2.1.1. Membrane fluidity and gel-to-liquid transition temperature (T_m)

Classically, membrane fluidity was first discovered by EPR spectroscopy and found to vary along the bilayer normal with different profiles, at a given temperature, according to the fatty acid chemical nature, as measured by the order parameter S [26]. At a fixed C-position, fluidity also changes with temperature [26] and in many membrane models, the trend of the order parameter S features a sharp fluidity
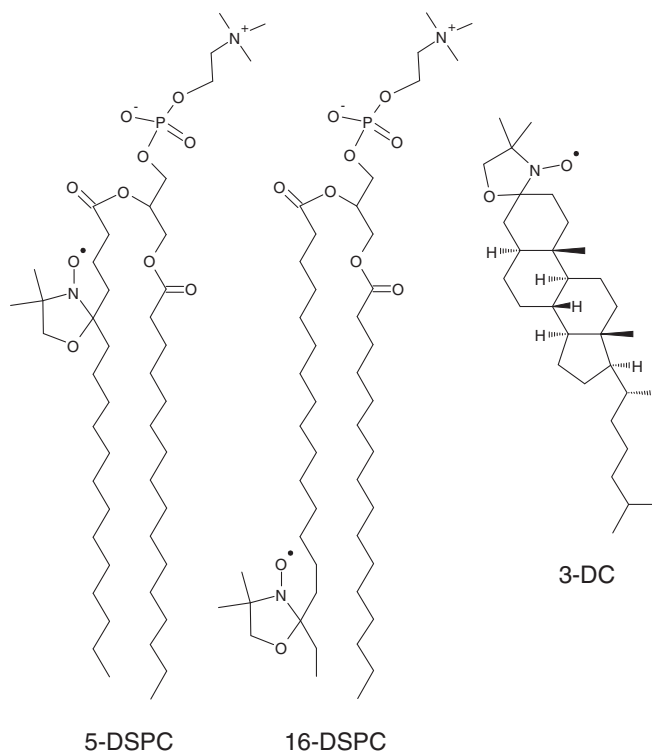

Fig. 1. Chemical structures of the EPR lipid probes used in the studies discussed in the present review.

increase at the gel-to-liquid transition temperature (T_m) typical of the bilayer fatty acid composition [27]. Until phospholipids are only considered, both membrane fluidity and T_m depend on the fatty acid unsaturation degree and chain length. When lipoperoxidation became of concern, the very first idea was that lipid oxidation, known to alter (or abolish) the double bonds arrangement of phospholipid poly-unsaturated fatty acids (PUFAs), would alter the bilayer fluidity, and that EPR spectroscopy would be able to detect lipid peroxidation via modification of typical S or T_m values of lipid spin labels, mainly n-doxylstearoyl-phosphatidylcholines (n-DSPC; see Fig. 1 for chemical structure) [10], as it is shown in Fig. 4 reporting the thermal behaviour of the hyperfine coupling constant (A_{max}) of 5-DSPC in the presence (panels A and B) and in the absence (panel C) of 25 mol% of class A oxPC in DPPC vesicles.

2.1.2. Fatty acid ordering

Fatty acid residues into the bilayer also share an average common orientation along the bilayer normal [26]. More precisely, owing to the fatty acid increasing segmental motion, fatty acids are pictured to move randomly into an ideal cone whose axis is aligned along the bilayer normal [26]. In case of restricted motion (as it occurs at lower C-positions) the fatty acid chains tend to be oriented along the bilayer normal, so that a geometrically ordered fatty acid ensemble can be envisioned. This picture applies fairly well to an oriented lipid bilayer on a solid support, usually referred to as supported planar bilayer (SPB), spin labelled with 5-DSPC [28]. Owing to the axial anisotropy of the nitroxide magnetic parameters g and A, two different EPR spectra are observed according to the orientation of the SPB normal with respect to the external magnetic field direction, based on the fact that the lipid spin label (5-DSPC) also shares the common orientation (EPR spectral anisotropy, see Fig. 6 in ref. [18], top spectra). Therefore, observation of two distinct EPR spectra upon changing the sample orientation betrays internal bilayer spatial ordering, while a geometrically disordered bilayer will yield orientation-invariant EPR spectra (spectral anisotropy loss, see Fig. 6 in ref. [18], bottom spectra). Also in SPBs it is expected that the presence of oxygenated groups, of conjugated dienes, and of cleaved fatty acids terminating with an aldehyde or carboxylic group, disturbs the fatty acid ordering, but in this case the consequent disordering of the spin label becomes visible as EPR spectral anisotropy change. This criterion was first proposed as a useful tool for the study of batch-oxidized phospholipid bilayers [29], and later applied to the more defined system, where the loss of EPR anisotropy was observed for the increasing molar percentage of certain oxPCs [18].

2.1.3. Polarity gradient and water penetration

The polarity profile is also an important feature of phospholipid bilayers due to water molecules penetrating into the bilayer, and its alteration reveals increased membrane permeability. Water penetration also depends on the phospholipid bilayer composition and is changed, as an example, by the mere presence of cholesterol [30]. The presence of polar groups of oxPLs can expectedly draw more water molecules than normal into the bilayer, bringing about a local polarity increase. The hyperfine coupling constant of a nitroxide (A_N) probe is sensitive to polarity in the sense that it increases in a polar environment, so the value of a nitroxide A_N can be used to gauge the bilayer polarity variation at a given C-position by use of n-DSPC [26].

2.1.4. Lateral phase separation

Hydrophobic mismatch originating from different fatty acid chain lengths often provokes segregation of different phosphatidylcholines in different membrane microdomains [31], giving rise to lateral phase separation. Beyond other techniques, the phenomenon is observable by EPR spectroscopy because the same spin label (particularly 16-DSPC, probing the methyl-terminal part of the bilayer) can yield a more or less fluid response according to its overspread location in both the terminal voids of shorter chains and close to the methyl-terminal of longer chains. In this case, the final EPR spectrum appears as a superimposition of two differently motion-restricted EPR spectra, revealing the existence of two differently fluid membrane domains [32,33]. Owing to their polar nature or to different lengths, oxPC-driven formation of membrane microdomains can thus be detected by 16-DSPC EPR spectra [34].

2.2. Fluorescence

Similar to the case of EPR, fluorescence techniques applied to lipid studies require introduction of a fluorophore into the system. This might be achieved by either incorporating an amphiphilic fluorescent dye that aggregates with the lipids due to the hydrophobic effect or by covalently attaching a fluorophore to the headgroup or hydrophobic chain of a lipid molecule. The consequences of fluorescent labelling of lipid aggregates are twofold. First, a word of warning: the presence of a fluorophore, which is usually bigger than the free radicals used in EPR, might interfere with the system of interest and change its properties. This should be taken into account when interpreting and designing fluorescence experiments. A careful choice of fluorescent dye and/or a crosscheck using different fluorescent labels is needed. Moreover, in view of the anisotropic nature of a lipid bilayer and its highly dynamical character, precise knowledge of the location of a fluorescent probe is mandatory. On the other hand, a continuously increasing number of fluorescent probes and labels together with the possibility of attaching them at different positions within the lipid bilayer give a great choice of parameters that can be measured at various specific locations. Fluorescent dyes used in the studies reviewed here are depicted in Fig. 2.

Modern fluorescence techniques allow the following: visualisation of lipid membranes and their lateral structure (including super-resolution techniques), localization and co-localization of fluorescently labelled species, determination of their diffusivity, fluidity, distances between them, measurements of physical and chemical properties of the system in their vicinity, and many others. A comprehensive list of fluorescent methods together with important considerations regarding their usage and fluorescence theory is given in [35]. Below, we shortly present those fluorescence methods that have been recently used to study oxidized model lipid membranes.

2.2.1. Fluorescence microscopy

Fluorescence microscopy provides a standard method of lipid bilayer imaging capable of capturing the lateral phase organisation. Application of phase-selective fluorescence dyes together with confocal scanning microscopy can picture phase domains in SPBs, giant unilamellar vesicles (GUVs), and lipid monolayers at the air/water interface [36]. Only recently, a number of methods have been used to improve the spatial resolution of fluorescence microscopy previously limited by Abbe's law of diffraction; today it can be as good as 20 nm [37].

2.2.2. Fluorescence correlation spectroscopy

Development of fast correlators and single-photon detection extended application of confocal microscopy allowed analysis of the dynamics of the system of interest. Fluctuations of fluorescence of diluted fluorescent molecules detected from very small confocal volume ($\sim 1\ \mu m^3$), when autocorrelated, give a direct measure of fluorophore concentration and dynamics. The technique is known as fluorescence correlation spectroscopy (FCS) [38]. In model membranes, it has been used mainly to measure the lateral diffusion of fluorescent lipid analogue. For this task, a number of FCS modifications have been made, starting with the so-called Z-scan FCS, which solved the problem of the need for precise focussing of the objective on the 2D sample [39]. Z-scan FCS allows the determination of lateral
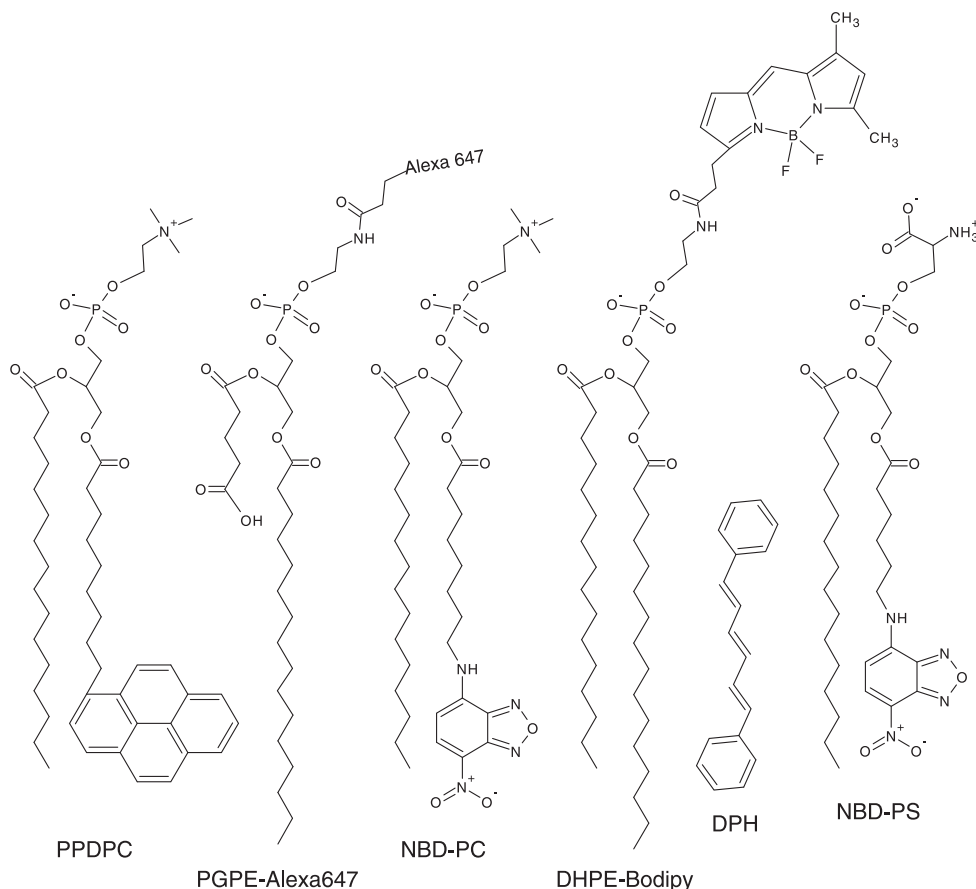
Fig. 2. Chemical structures of the fluorescent dyes used in the studies discussed in the present review. Fluorescent polarity probes used in TDFS method are shown in Fig. 7.

diffusion coefficient of lipids, but it can also be used to distinguish between free and hindered diffusion. The method has been applied for SPBs, GUVs, as well as for lipid monolayers.

2.2.3. Fluorescence quenching and Förster resonance energy transfer

Fluorescence quenching and Förster resonance energy transfer (FRET) allow localisation of a fluorophore in relation to the location of its quencher (or FRET partner) molecule. Both techniques, while simple in their principles, are extremely versatile in studying the structure of lipid membranes and their interactions with proteins and other compounds. An interesting example of fluorescence quenching application is a chemical quenching of NBD lipid analogue by dithionite. Since water-soluble dithionite cannot diffuse trough a lipid bilayer, it can merely quench the NBD dyes in the outer leaflet (single monolayer) of the bilayer when added to the liposomal suspension. This results in an asymmetrically labelled bilayer, for which the kinetics of lipid flip-flop (hopping of the lipid molecule from one leaflet to the other) can be studied by simply following the fluorescence intensity decrease upon NBD-PC flopping from the inner liposome leaflet where it was not affected by dithionite to the outer leaflet, where it is being irreversibly quenched.

2.2.4. Fluorescence anisotropy

Membrane fluidity, defined as reciprocal to the lipid structural order parameter *S*, can be derived from fluorescence polarity measurements [40]. The most commonly used fluorescent probes for this purpose are DPH and its derivatives. Located at the hydrophobic backbone of the lipid bilayer DPH is wobbling, which decreases the measured fluorescence anisotropy. The wobbling diffusion rate was shown to be proportional to the local fluidity. An interesting technique that allows measurement of both structural and dynamic

properties of oriented planar bilayers using only steady-state instrumentation is the so-called angle-resolved fluorescence depolarization (AFD) method [41]. While fluidity is a very useful measure of local membrane dynamics/packing in the backbone region and especially for gel phase bilayers, for which the anisotropy is high, it is less suitable for probing the head group mobility of liquid–crystalline bilayers, for which the method of choice is the time-dependent fluorescence shift technique.

2.2.5. Time-dependent fluorescence shift

Fluorescence solvent relaxation or time-dependent fluorescence shift (TDFS) is based on the sensitivity of certain fluorescent polarity probes to the relaxation of their polar surrounding [42]. When applied to lipid membranes, it is capable of probing hydration and mobility at a defined level of the fully hydrated free-standing liquid–crystalline bilayers, which are the most physiologically relevant lipid systems [43]. Time-resolved fluorescence decays measured at different emission wavelengths serve to reconstruct a time-resolved emission spectra (TRES). The position of TRES, $\nu(t)$, is shifting in time toward lower energies. It was shown that the total spectral shift, $\Delta\nu$, is proportional to membrane hydration, and the kinetics of relaxation, often described as mean relaxation time, τ, is reversely proportional to the local dynamics of the hydrated lipids. The method has proved its usefulness in a number of studies being usually more sensitive to small changes in lipid packing than the lateral diffusion measured using FCS. A set of fluorescent polarity probes precisely located at different depths across the lipid bilayer allows probing the whole profiles of the bilayer hydration and mobility; see the example in Fig. 7. It is also relatively easy to link TDFS method with computer simulations, which allows molecular interpretation of the observed changes [44].

2.3. Molecular dynamics simulations

Molecular dynamics (MD) simulations applied to lipid membranes provide an atomistic-level description of the system. Positions of individual atoms in the system are followed by numerically solving classical equations of motion. Potential energy of the interactions is described in the form of force-field, based on both empirical and quantum chemical data. As a matter of fact, given that the chemical and physical parameters of the molecules involved are correct and able to precisely describe their behaviour, those simulations can be considered as a "magnifying glass" with an unprecedented spatial resolution of the membrane under scrutiny. MD simulations also include a detailed description of the dynamic of the molecules studied, and the values of the correlation time and of the order parameter calculated by the method at the various C-positions almost always match those obtained by classic methods (NMR, EPR, fluorescence) very well. Most impressively, also other bilayer features, such as thickness and cross-sectional distribution of atoms along the bilayer's normal obtained by MD simulation closely match the data obtained by X-rays or neutron beam diffraction [22,45]. Thus, MD simulations may serve as a technique complementary to the experimental fluorescence and EPR methods in which more coarse-grained system properties are obtained. This issue is particularly important in the case of oxidized phospholipid bilayers, as many of the changes with respect to non-oxidized systems originate from the alteration of molecular configurations due to membrane oxidation. The main effects include conformational changes of oxPL molecules and increased water penetration into the membrane, the latter assisted by the formation of new hydrogen bonds. To address these phenomena at an atomistic-level of description, the united-atom force-field based on the Berger's force-field was employed in MD simulations of oxidized membranes [46]. The parameterization for oxPC lipids was performed by the group of Tieleman in their pioneering MD study of oxidized membrane [45]. In one case, the all-atom force-field was also employed, but no qualitative differences from results obtained using a united-atom force-field were noted [47]. The coarse-grained MARTINI force-field [48] was used in a recent study of the influence of cholesterol on oxidized bilayer allowing for simulations of long-timescale behaviour of lipids and estimation of lateral diffusion coefficients in a membrane consisting of 5000 lipid molecules [49].

In the studies presented here, the MD results were directly comparable with fluorescence experiments in terms of hydration of the bilayer, lateral diffusivity, and free energy barrier of lipid flip-flop. Indirect comparison included conformational changes of lipids, area per lipid, and membrane thickness. In all cases, at least semi-quantitative

agreement was achieved. Some limitations of MD methods, important from the point of view of oxidized membranes, should be noted. First, the timescale of the considered processes is limited to hundreds of nanoseconds. To some extent, this issue can be overcome by employing free-energy simulation techniques; however, long-time changes in the bilayer structure cannot be fully addressed yet. Second, the size of the simulated membrane is limited to several nanometres. Thus, no phenomena happening at longer length scales can be studied, which includes, for instance, the creation of large water defects and pores.

3. Studies on lipid membranes containing chemically defined oxidized phospholipids

Within the recent years, several studies have appeared focusing on the influence of oxidized phosphatidylcholines on the biophysical properties of model membranes. Chemical structures of oxPCs, whose effect on the biophysics of lipid bilayers was investigated, are depicted in Fig. 3. We decided to distinguish two classes of oxidation products that were studied using EPR, fluorescence or MD simulations: class A — hydroxy- or hydroperoxy-dieonyl oxPCs (Fig. 3A), class B — oxPCs with the truncated chains (Fig. 3B). Two members of the class B: ox1-DOPC with truncated sn-1 chain and ox2-DOPC with both chains truncated, have not been found in Eukaryota, since majority of their phospholipids carry saturated sn-1 chains, which do not oxidize. They were, however, studied in silico as theoretical products of DOPC oxidation, and thus, are included in this review.

3.1. EPR

A comparative EPR study of the effects exerted on the lipid bilayer by different oxidized PC species, probed with 5-DSPC [18], showed that oxidized conjugated diene species (Fig. 3A) were unable to disorder the phospholipid bilayer, while extremely oxidized cleaved chain PC (Fig. 3B) were revealed to be responsible for the previously observed strong EPR anisotropy loss induced by Fenton-oxidized phosphatidylcholine [29,34].

This study confirmed the need of well defined pure molecular species of oxPC for detailed studies of oxidized bilayers.

3.1.1. EPR studies using 5-DSPC and 16-DSPC spin labels in SPB and MLV

More systematic EPR studies were performed using four defined oxidized PC molecular species (HPPLPC, HOPLPC, and the truncated lipids: PGPC, and PAzPC; see Fig. 3) in hope of attaining a better definition of structural changes in MLVs owing to the sharp definition of
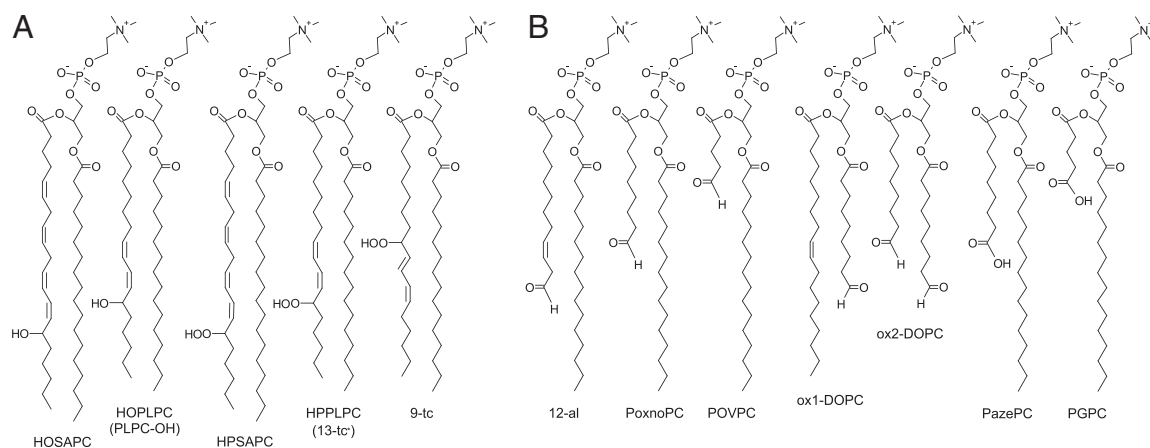
Fig. 3. Chemical structures of oxidized phosphatidylcholines (oxPCs). (A) Hydroxy- (HOSAPC and HOPLPC) and hydroperoxy-(HPSAPC, HPPLPC, and 9-tc) phospatidylcholines. Different cis/trans isomers are possible. *13-tc refers to trans-11,cis-9 isomer of HPPLPC. (B) Truncated (cleaved chain) phosphatidylcholines with aldehyde (12-al, PONPC, POVPC, ox1-DOPC, and ox2-DOPC) and carboxylic (PAzPC and PGPC), functional groups. Please note that the two aldehyde-truncated oxPCs studied in silico (ox1-DOPC, and ox2-DOPC) have their sn-1 chains truncated, in opposition to all the other oxPCs presented in the figure.

the oxidized molecules used [14]. This study also indicated that cleaved chain (truncated) PCs (Fig. 3B), especially the ω-carboxyacyl-species, were able to give rise to micelle formation from the lipid mixtures they were enclosed in, in agreement with a much earlier advanced hypothesis [15]. Therefore, that study was limited to full-chain conjugated diene PCs, with proven vesicle-forming ability (Fig. 3A). The results of this study clearly indicated that conjugate diene-PC/DPPC MLVs (1:3 mole ratio) featured lateral phase separation at room temperature and T_m value lower than that of pure DPPC MLVs, yet higher than that of 1:3 PLPC/DPPC and closer to T_m of 1:9 PLPC/DPPC (Fig. 4). Pure conjugated diene-PC MLVs resembled more pure PLPC MLVs, and in mixed MLVs they displayed free miscibility with PLPC. In more detail, in the temperature range of 5 °C to 45 °C, HPPLPC MLV bilayer appeared to be slightly more rigid than that of HOPLPC's, although this difference was less visible in 1:1 mixtures with PLPC. Nonetheless, the polarity profile of MLVs made of any of the considered pure conjugated diene species was similar to that of normal PLPC. This property is linked to water penetration into the bilayer and cross-membrane water permeation. Its variations due to the presence of OXPC in the bilayer is worth deeper studies, e.g. by W-band EPR spectroscopy.

In summary, the tendency of class B oxPCs to form micelles was confirmed and lateral phase separation and T_m alterations were
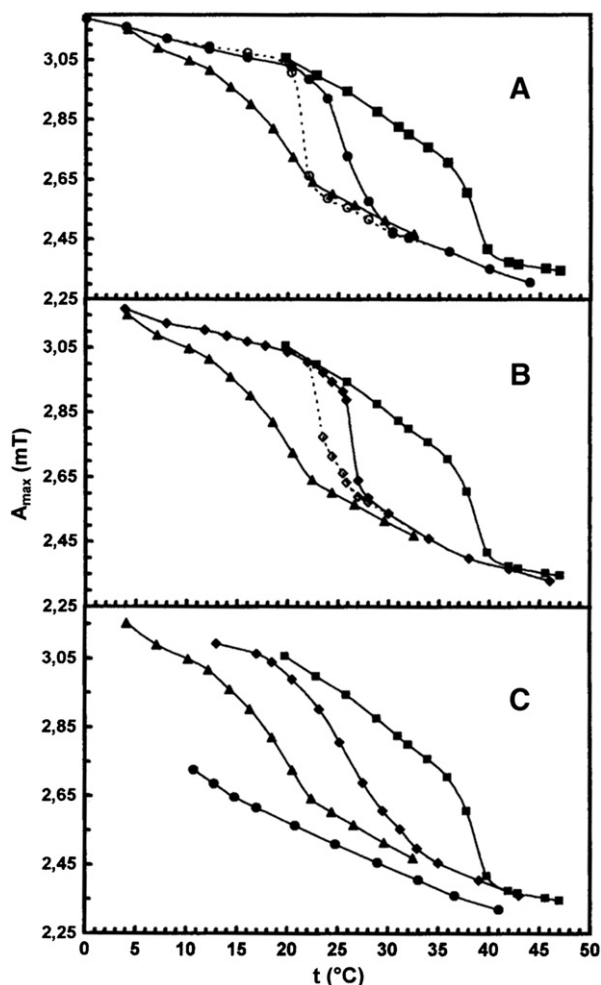
observed for the first time. The latter were better defined than membrane fluidity variations. It has been clearly established that alterations of phospholipid bilayer structural properties do correlate with the presence of class A oxPCs.

3.1.2. Comparative EPR studies using 5-DSPC and 3-DC spin labels in SPBs

The inability of conjugated dienes PC to disorder the bilayer fatty acids, as revealed by 5-DSPC in supported lipid bilayers (SPBs), was subjected to a deeper study performed by the use of a different spin label, 3-DoxylCholestane (3-DC) [50]. This spin label is a y-rotor, and allows probing the bilayer under a different perspective than 5-DSPC (a z-rotor). The two spin labels were used comparatively to determine the disordering capacity of up to all eight oxidized PC molecular species shown in Fig. 3 (class A: HOSAPC, HOPLPC, HPSAPC, and HPPLPC; class B: PONPC, POVPC, PAzPC, and PGPC). The response of 3-DC to the presence of truncated (cleaved chain) PCs was a heavy loss of EPR spectral anisotropy, similar to that shown by 5-DSPC. Nonetheless, 3-DC also reported disordering from conjugated dienes PCs (class A oxPC, Fig. 3A), not revealed by 5-DSPC. The different sensibility of the two spin labels was attributed to their different chemistry more than to different bilayer structural features. 3-DC nitroxide group is reported to localise close to the phospholipid polar head region, with the cholesterol hydrophobic frame buried into the fatty acid chain ensemble. Since the cholesterol – OH group is replaced by the less polar nitroxide ring no strong interaction stabilizes this localization. Strongly polar hydroxy- and hydroperoxy-groups of class A oxPCs are reported to come close to the polar head region due to hydrogen bonding and polar interaction, so that they could displace unbound 3-DC and make it move to a more fluid inner bilayer region. As a matter of fact, EPR spectral parameters of this probe in oxidized SPBs favoured a fairly free tumbling more than a rigid disoriented probe. In contrast, 5-DSA residue in 5-DSPC is firmly bound to the phospholipid molecule and stably assembled in a distended configuration into the bilayer, so its displacement is expectedly harder than 3-DCs. In support of this explanation, EPR spectra of 5-DSA and of its methyl ester in MLVs were compared. The former, capable of interacting with the bilayer polar region via the free carboxyl group, showed rigid EPR spectra very similar to 5-DSPCs, while the latter, in which the interaction possibility is suppressed, yielded EPR spectra reporting much higher fluidity, suggesting the possibility of a displacement of this weakly interacting spin label to a differently fluid bilayer region. Therefore, results of this study were taken as an indication that 3-DC is useful more for revealing the mere presence of all perturbing oxidized PC molecules than for gaining information about structural changes. The question of the ordering maintenance of 5-DSPC in the presence of CD-PCs remains unanswered to date.

In summary, although 3-DC is sensitive to the presence of both class A and B oxPCs, it is still uncertain whether it is able to report variations of the bilayer organisation. However, experiments using 3-DC confirm that class B oxPC disorders the lipid bilayer, similar to what was revealed by 5-DSPC.

3.1.3. Combining EPR studies with gel chromatography and cryo-electron microscopy

In a recent study we addressed the issue of micelle forming tendency of cleaved chain PCs. Transition of vesicles to micelles upon addition of oxPC is a dramatic event with heavy consequences for the membranes of living cells, ending in membrane local rupture and full breakdown. Amongst natural phospholipids, micelle forming ability of lyso-phosphatidylcholine has been studied most intensively. Soon after, the lysolecithin antagonist capacity of cholesterol was demonstrated. Since micelle forming capacity of cleaved chain oxPCs (class B), similar to that of lyso-PCs, was previously observed the issue of the antagonist capacity of cholesterol against oxPC membrane disruption was also addressed [51]. The analysis of EPR

Fig. 4. EPR detection of the lipid phase transition temperature, T_m, in multilamellar vesicles (MLV). Panel A: ■ pure DPPC, ▲ DPPC/PLPC 3:1[*], ● DPPC/HOPLPC 3:1[*] spectral component I, ○ DPPC/HOPLPC 3:1[*] spectral component II. Panel B: ■ pure DPPC, ▲ DPPC/PLPC 3:1[*], ◆ DPPC/HPPLPC 3:1[*] spectral component I, ◇ DPPC/HPPLPC 3:1[*] spectral component II. Panel C: ■ pure DPPC, ◆ DPPCIPLPC 9:1[*], ▲ DPPC/PLPC 3:1[*], ● pure PLPC. [*] (mol:mol).
Adapted from [14].

anisotropy loss of spin labelled oriented bilayers was useful in establishing the increasing bilayer destabilisation with increasing class B oxPC molar ratio, as well as the reversal of this destabilisation when 40 mol% cholesterol was included in the preparation. Nonetheless, the EPR method was unable to distinguish between the presence of scrambled chains bilayers and formation of micelles solely on the base of EPR anisotropy loss of spin labelled (3-DC or 5-DSPC) oriented bilayers. A correct attribution of chain geometrical disordering to disordered bilayers was achieved by complementing EPR observations with morphological analysis of lipid suspensions by Sepharose 4B gel chromatography. The method is useful to assess the bilayer forming ability of a given phospholipid by distinguishing between the formation of vesicles (MLV) and micelles. This criterion helped in demonstrating that PGPC (and, expectedly, class B oxPCs, in general) was able to form micelles in the absence of cholesterol, while, when the latter was included, bilayers were obtained (Fig. 5). After establishing the lamellar structure, the inner chain disordering was confirmed by EPR spectral anisotropy of PGPC-containing planar bilayers. Definitive confirmation of oxPC/cholesterol vesicle formation was achieved by cryo-electron microscopy (Fig. 5), though EPR anisotropy confirmed that internal bilayer order was not recovered. On the contrary, class A oxPC (HOPLPC) always yielded MLV (indicated by gel-chromatography) with different internal ordering degrees (as indicated by EPR anisotropy of planar samples), depending on the amount of HOPLPC content and on cholesterol presence.

In summary, EPR data indicated a loss of ordered lamellar structure and micelle formation brought about by class B oxPC, while gel-chromatography and cryo-EM allowed demonstrating the ability of cholesterol to reshape class B oxPC micelles into unilamellar (disordered) structures. EPR anisotropy also showed the cholesterol ability to restore internal bilayer ordering in class A oxPC lamellar structures.

3.2. Fluorescence

3.2.1. Angle-resolved fluorescence depolarization technique (AFD)

Information on structural organisation and molecular dynamics within a planar lipid bilayer can be gained by means of the angle-resolved fluorescence depolarization technique (AFD). Wratten and co-workers studied bilayers that consisted of class A oxPCs (HPPLPC or HOPLPC) and phosphatilylcholines (PLPC or DLPC) using angle-resolved A-ESR and AFD [17]. AFD measurements were performed with DPH introduced in macroscopically oriented planar bilayers containing 2 to 5 mol% of oxPCs. The analysis of the AFD measurements showed that increasing amounts of oxPCs in the PLPC membranes

Fig. 5. Elution profile of Sepharose 4B gel-chromatography of aqueous suspensions of: 100% PGPC (dashed line); 2:1 PGPC/PLPC (dotted line); PGPC/C 3:2 (solid line). The insets show the cryo-electron micrographs of the content of the corresponding elution peak.
Adapted from [51].

result in an overall decrease in molecular orientational order as defined by the order parameters of DPH in the bilayers, however, the reorientational dynamics of DPH appeared not to be affected. These results of AFD were supported by those of A-ESR. Despite the fact that DPH and 3-DC used in A-ESR have different locations within the membrane, they gave similar results with respect to membrane order and dynamics.

In summary, DPH fluorescence anisotropy study indicates a decrease in the overall order within phospatidylcholine bilayers induced by the presence of class A oxPCs.

3.2.2. Visualization — microscopy/monolayer studies

The influence of two oxPCs (PONPC and PAzPC, Fig. 3B) on the lateral pressure and phase organisation of DPPC monolayers was studied by Sabatini and co-workers using Langmuir balance and fluorescence spectroscopy [52]. An analysis of the compression force-area isotherms for pure DPPC film reveals the liquid-expanded (LE) to liquid-condensed (LC) phase transition with LE–LC coexistence region. Isotherms collected for mixtures of PONPC with DPPC or PAzPC with DPPC show different behaviours. With increasing fraction of PONPC in DPPC monolayer, LE–LC coexistence region progressively vanishes, and almost completely disappears at 40 mol% of PONPC. These results suggest that films with more than 40 mol% PONPC are predominantly in the LE phase. Similar results were obtained for PAzPC/DPPC monolayers, but the disappearance of LE–LC coexistence region is visible at smaller PAzPC fractions. The expansion of the monolayers can be explained by the looping back of sn-2 acyl chains of PONPC and PAzPC with their aldehyde and carboxylic moieties, respectively, accommodating in the vicinity of the lipid headgroups. The stronger influence of PAzPC might be explained by the negative charge, higher affinity for water and larger size of carboxylic moiety in comparison to that of the PONPC aldehyde. At higher pressure (~42 mN/m) a discontinuity in the isotherms was observed for all oxPC-containing monolayers. A plausible explanation provided by the authors was a hypothesis of a complete extension of the oxidized sn-2 chains into water (so-called extended lipid conformation), followed by the dissolving of the oxPC in the aqueous subphase.

Fluorescence microscopy experiments using fluorescent lipid analogue, NBD-PC (2 mol%) which favours the liquid-expanded (LE) phase, were performed to visualize the lateral phase organisation of the monolayers studied. For pure DPPC monolayers non-fluorescent solid domains were observed in the LE–LC coexistence region, while at higher pressures the boundaries of the domain became blurred and NBD-PC crystallised into clusters appearing as bright white spots. Addition of PONPC or PAzPC caused the NBD-enriched domains to disappear and shifted the appearance of LC domains to higher surface pressures. In the case of PAzPC/DPPC monolayers, a smaller number and larger sizes of the domains were observed in comparison with the PoxnoPC/DPPC system. Moreover, the author postulated that lateral segregation occurred for PAzPC in its extended conformation, but not for PONPC.

In summary, a possible looping-back of the truncated oxidized chains and the so-called extended conformation of class B oxPC were postulated for the first time. Fluorescence microscopy showed that oxidation can alter the phase behaviour of PC monolayers.

3.2.3. FRET

The FRET method was used to study the conformational dynamics of class B oxPCs (PAzPC and PONPC) [53]. The system of interest consisted of pyrene containing phospholipid analogue PPDPC added to oxPC micelles and a water soluble cationic protein, cytochrome c. The analysis of the distance-dependent efficiency of Förster-type resonance energy transfer between those two molecules provided important information on the conformational dynamics of the oxPC. Cytochrome c was found to bind to the PAzPC micelles, but only weakly associate with PONPC. Based on those results, a model of
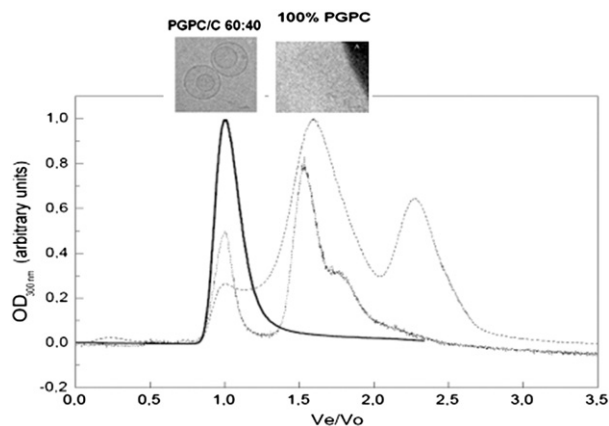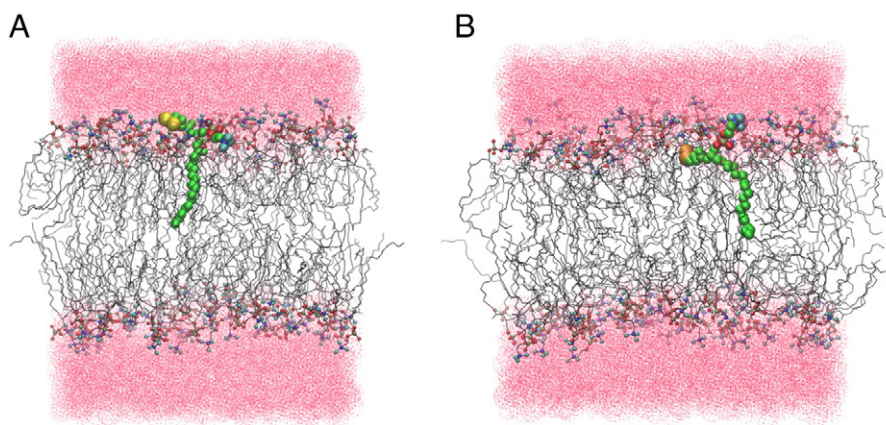
Fig. 6. Typical configurations of PGPC (A) and POVPC (B) lipids in the POPC membrane (based on MD trajectories from [54]). The truncated *sn*-2 chain of PGPC typically protrudes into the water phase whereas in the *sn*-2 chain of POVPC resides in the head groups region. Colour coding: oxidized lipids — green balls, POPC — black lines, choline groups — small blue balls, phosphate groups — small yellow and red balls. Polar terminal groups of *sn*-2 chain of OXPCs are depicted as yellow (PGPC) and orange (POVPC) balls.

cytochrome c–oxPC interaction was proposed. According to that, the looping-back truncated chain of PAzPC exposes its carboxyl function on the micelle surface where it can readily bind cytochrome c. This is not the case for PONPC the aldehyde-truncated *sn*-2 chain of which did not bind to cytochrome c.

In summary, the energy transfer between a peripheral membrane protein and fluorescently labelled class B oxPC micelles revealed, for the first time, an important difference between aldehyde- and carboxy-truncated oxPCs and confirmed the hypothesis that carboxy-truncated lipids might loop back towards the water phase (see Fig. 6 for illustration).

3.2.4. FCS

To better understand the behaviour of class B oxPCs in membranes, well defined model systems were studied by Plochberger and co-workers using FCS [49]. The lateral diffusion of oxPC fluorescent analogue, PGPE-Alexa647, in various SPBs was compared to that of conventional lipid analogue, DHPE-Bodipy. Diffusion constants obtained from line-scan FCS experiments were found to be considerably higher for PGPE-Alexa647 than for DHPE-Bodipy which means that the oxPC was more mobile than non-oxidized lipid. An insertion of immobile obstacles blocked the diffusion of DHPE-Bodipy while the diffusion of PGPE-Alexa647 was only slightly affected. Moreover, the addition of 40 mol% of cholesterol resulted in slowing down and levelling the diffusion of both probes in DOPC SPBs. Based on the results of coarse-grained simulations (see Section 3.3), this effect was ascribed to a deeper incorporation of PGPC into DOPC bilayer in the presence of cholesterol.

The lateral diffusion of a fluorescent lipid analogue, Bodipy C_{12}-HPC in oxPC-containing SPBs was also measured by Beranova and co-workers. Z-scan FCS measurements of lateral diffusion of Bodipy C_{12}-HPC in SPBs composed of POPC with 10 mol% of short-chained truncated oxPCs (PGPC or POVPC, Fig. 3B) revealed faster lateral diffusion in both oxPL-containing bilayers. These results were compared with the results from TDFS experiments and MD simulations and are discussed later in the text.

In summary, FCS reports considerably faster diffusion of the labelled oxidized lipid (PGPE) in SPB. Moreover, this diffusion was almost not affected by the presence of immobile obstacles but slowed down and levelled with increasing cholesterol content. The latter finding indicated an interplay between oxPL and cholesterol as already pointed out in Section 3.1.3.

On the other hand, the diffusion coefficients of labelled non-oxidized PC were only slightly increased in the presence of both class A oxPCs.

3.2.5. Time-resolved fluorescence shift

As mentioned in Section 2.2.5, time-resolved fluorescence of certain polarity probes gives us a measure of lipid membrane hydration and local mobility. In the work of Beranova and co-workers [54] Laurdan – a fluorescent dye of precisely known location in the phosphocholine bilayer (see Fig. 7) [55,56] – was used to probe hydration and mobility of hydrated carbonyls of POPC bilayer with 10 mol% of class B oxPCs (POVPC and PGPC). It was shown that both aldehyde and carboxyl at their *sn*-2 chains (POVPC and PGPC, respectively) considerably disturb the structure of model phosphatidylcholine membrane introducing voids in the hydrocarbon region of the bilayer and polar functional groups (aldehyde and carboxylic) that are looping back toward the aqueous solution. These structural changes profoundly affect the parameters probed by Laurdan. Lipid carbonyls become much more hydrated and mobile. While the effects are apparent for both PGPC and POVPC, more dramatic effects are observed for the aldehyde-truncated lipid (POVPC). These findings point out that not only chain truncation and resulting conical geometry of the studied oxPL molecules but also the chemical character of the truncated chain can alter the mechanistic properties of the oxidized lipid bilayer. Thus, the different biological functions that the two lipids have [57] can also be governed through the biophysics of the lipid bilayer apart from the specific receptor recognition.

When compared with lateral diffusion measurements performed using FCS, local mobility measured using fluorescent polarity probes proved to be much more sensitive to the presence of truncated oxPLs. This is an advantage of probing membrane properties locally.

In summary, addition of 10 mol% of class B oxPCs strongly increases POPC carbonyl region hydration and local mobility. The effects are stronger for the aldehyde (POVPC) than for carboxylic acid (PGPC).

3.2.6. Polarity profiles

A series of fluorescent polarity probes located at different depths of the lipid bilayer allows using the time-dependent fluorescent shift method to probe the whole profile of membrane polarity in the direction perpendicular to its surface. The finding, discussed in Section 3.2.7, that the addition of PAzPC or PONPC largely facilitates flip-flop of phosphatidylserine (PS) in POPC bilayer led to the question of the possible reason for this effect. It is commonly agreed as well as calculated (see Section 3.3) that the main energetic barrier for the phospholipid molecule moving from one leaflet of the bilayer to another is given by the hydrophobicity of the bilayer interior, through which the polar serine group of PS has to cross. This reasoning motivated the investigation of polarity profiles in POPC bilayer
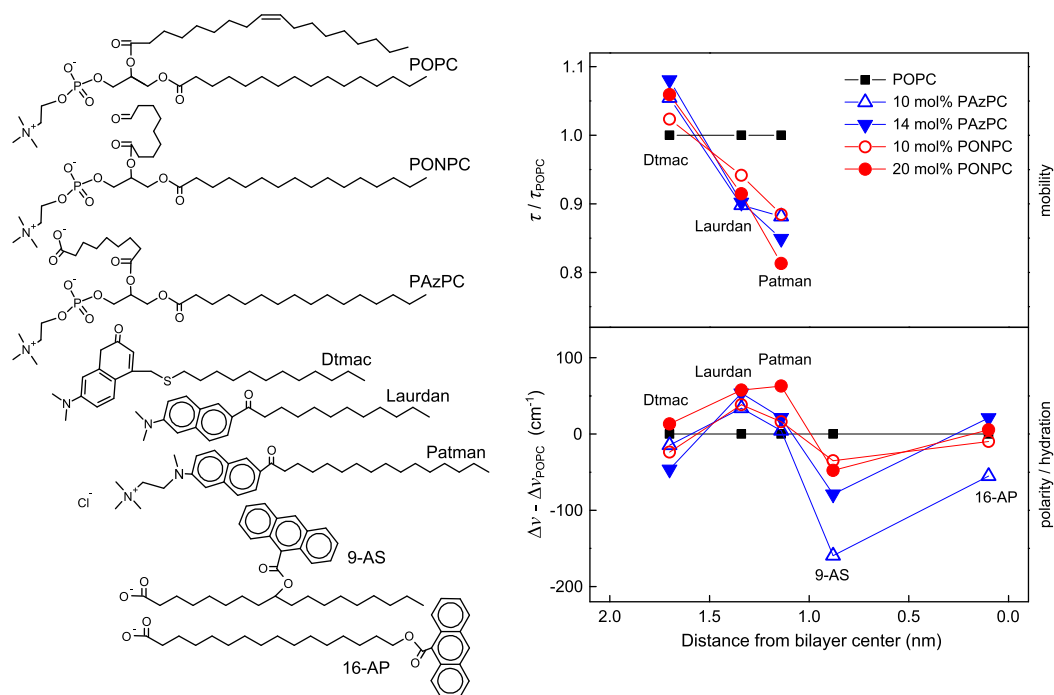
Fig. 7. Relative polarity/mobility profiles of oxPC-containing POPC bilayer. TDFS parameters relative integrated relaxation time (A) and differences in spectral shift (B), normalised to the values obtained for pure POPC. Structures of polarity probes are shown at their positions known from literature (see the text). For the sake of clarity of presentation the position of Laurdan has been shifted by 0.1 nm outward from Patman.

upon addition of PAzPC or PONPC. Polarity and mobility changes have been measured at 5 different depths of the bilayer using the following fluorescent polarity probes (ordered from the most outside to the most inside locations): Dtmac, Laurdan, Patman, 9-AS, 16-AP [58]. The structures of the probes, their location in the lipid bilayer and the results obtained are shown in Fig. 7. In agreement with the previous studies [54], discussed in Section 3.2.5, Laurdan and Patman report significantly increased the polarity and mobility at the level of lipid carbonyls upon addition of both PAzPC and PONPC (the effects of both oxPCs are similar). The polarity and mobility measured for Dtmac and the polarity measured for 9-AS and 16-AP show opposite changes to those of Laurdan and Patman. The environment of Dtmac located at the phosphate group is probably affected by the presence of truncated oxidized chains that are looping back toward the aqueous solution. Their presence slightly restricts both mobility and hydration of this region.

There is a noticeable decrease in polarity for both 9-AS and 16-AP located roughly at 9th and 16th carbons of the lipid hydrocarbon chains. Unfortunately, due to complex photophysics of those dyes, a precise calculation of the relaxation time has been impossible and thus no information on probable mobility alterations can be given. The overall changes in membrane polarity profiles in oxidized bilayers, relative to polarity profiles obtained for pure POPC, have a sinusoidal shape (see Fig. 7). An explanation for the decreased polarity of the membrane interior probed by 9-AS and 16-AP might be the thinning of the bilayer upon addition of oxPL observed in MD simulations [58]. This might cause a restriction of mobility of the fluorophores buried in tighter packed hydrocarbon chains (higher total density in the centre of oxidized bilayer as seen in Fig. 6B in [58]). Altogether, even the polarity of the membrane interior does not increase, water penetrates much deeper (increased hydration at the carbonyls) and membrane becomes thinner, all of which leads to the dramatic increase of the observed PS flip-flop.

In summary, the presence of class B oxPCs significantly changes hydration and thus polarity of the bilayer, which is likely to contribute to the faster PS flip-flop in oxidized model lipid membranes.

3.2.7. Dithionite quenching

Maintaining the lipid bilayer compositional asymmetry is known to be important for cell physiology and a failure to fulfil this requirement can lead to cell death by triggering the so-called programmed cell death (apoptosis). One of the distinctive signs of cell malfunction that can be recognized by the appropriate receptors resulting in cell apoptosis is the presence of phosphatidylserine (PS) molecules in the outer leaflet of the cellular membrane. In a healthy cell, PS is kept in the inner leaflet of the bilayer as a result of an equilibrium of the action of ATP-dependent substrate-specific transporters on the one side and the flip-flop — an entropy-driven process of lipid randomization (cross bilayer diffusion of lipids) on the other side. It was proposed that the flip-flop might be facilitated by the action of a protein called scramblase [59], which has not been identified so far.

The recent fluorescence quenching studies have shown that the presence of class B truncated oxPCs (namely PAzPC and PONPC) can radically raise the flip-flop rate in a model membrane [58]. The kinetics of flip-flop of the NBD labelled PS has been followed using a dithionite-quenching assay [60]. Dithionite is water-soluble but does not permeate through lipid membrane and when added to liposomal suspension, it chemically quenches NBD-labelled lipids in the outer leaflet of the bilayer only. The measured attenuations in the extent of decrease in fluorescence upon the external addition of dithionite have shown that the presence of 14 mol% of PAzPC or 16 mol% of PONPC in POPC bilayer brings the PS flip-flop half-time from weeks down to hours. These results demonstrate that a passive biophysical mechanism might be sufficient for the loss of lipid compositional asymmetry, i.e. a scramblase protein might not be needed.

In summary, class B truncated oxPCs increase lipid flip-flop rate.

3.3. Molecular dynamics simulations

3.3.1. Structure and lateral mobility

Molecular dynamics studies of oxidized phospholipid bilayers were initiated by Wong-ekkabut and co-workers [45]. Properties of a PLPC (1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphatidylcholine) membrane with varying amounts of different oxidation products

were investigated. As the products, four oxidized phospholipids derived from the following fatty acids were considered and used as a replacement of sn-2 chain in PLPC: 13-hydroperoxy-trans-11, cis-octadecadienoic acid (13-tc, i.e., trans-11,cis-9 isomer of HPPLPC), 9-hydroperoxy-trans-10,cis-12-octadecadienoic acid (thus, forming HPPLC lipid9-tc, an analogue of 13-tc with a hydroperoxy group located at the position of 9th carbon in the sn-2 chain), 9-oxo-nonanoic acid (PONPC), and 12-oxo-cis-9-dodecenoic acid (12-al). These acids are products of linoleic acid peroxidation, based on a previous theoretical study [61]. Thus, four oxidation products, with either aldehyde or hydroperoxide group at the terminated sn-2 chain, and with two different localizations of polar moieties in the oxidized chain were accounted for. Oxidation ratios between 2.8 and 50% were considered.

The main effect observed for oxidized bilayer was the bending of oxidized tails toward the water phase. The polar groups of oxidized chains formed hydrogen bonds, predominantly with water and, to a lesser extent, with lipid headgroups. This reorientation was stronger for lipids containing hydroperoxide groups due to a higher hydrophilicity of hydroperoxide with respect to that of the aldehyde group. The changes in conformation of oxidized chains led to increased area per lipid (APL) by up to 10% in the case of 50% of oxidation. APL increased monotonously with the increasing content of oxidized lipids. Conformational changes in the oxidized membrane caused reduction of the deuterium order parameter of lipids with respect to pure-PLPC membrane; this effect was stronger in the presence of aldehyde-terminated lipids than in the case of acid-terminated ones. Changes in electron density were also observed, namely, an increased density in the membrane interior and a decreased density in the headgroup region, accompanied by a shift of the head group peaks toward the membrane interior. Membrane thickness was generally reduced with increasing oxidation ratio. No demixing of lipids was observed within the simulation timescale and lateral diffusivity was not affected by oxidation. The creation of water defects, which were larger than in the non-oxidized membrane, was observed due to reorientation of oxidized chains. A free-energy barrier of water permeation estimated employing the PMF method was reduced by up to 10 kcal/mol (from ~29 kcal/mol) in the case of 50% oxidized bilayer. The main conclusion given in the paper is that the cell membrane damage which accompanies oxidation is caused by increased membrane permeability for water. This result is in accord with early experimental studies of the leakage of oxidized liposomal membranes [62,63].

In the following study, Khandelia and Mouritsen investigated properties of POPC bilayer with up to 25% of oxidized lipids, either PONPC or PAzPC [47]. These two lipids were chosen as stable products of lipid oxidation under physiological conditions [64,65]. A complete reversal of orientation of the anionic PAzPC chains, without a loss of bilayer integrity for up to 25% content of PAzPC was observed, with the reversal being more pronounced in higher PAzPC content. The carboxylic groups were able to extend up to 1 nm into the water phase beyond the average position phosphorous atoms. This was the first direct observation of the so-called "extended lipid conformation" suggested previously by Kinnunen [66]. For neutral PONPC, the chain reversal, although not complete, was also observed. An incomplete reversal of aldehyde-terminated chains caused stronger perturbation of the bilayer structure than in the case of PAzPC. Note, however, that for comparisons between the membrane with neutral PONPC and anionic PAzPC the presence of neutralising sodium counterions in the latter system must be accounted for as a factor introducing extra stabilization of the bilayer. Alteration of electron density upon oxidation was observed, with a density increase in the centre of the bilayer and a shift of its maxima toward the centre. For PAzPC a small increase of the density in the aqueous region due to the sn-2 chain reversal was present. APL for PONPC increased, whereas for PAzPC a reduction of APL was observed, the latter resulting

from the presence of sodium ions in the system (sodium cations strongly bind carbonyl groups of lipids causing a reduction of APL [67]). A decrease of membrane thickness was observed in both systems, being stronger for PONPC. The deuterium order parameter of lipids was reduced in oxidized bilayers with respect to PLPC membrane. No significant alteration of lateral diffusion was detected. Oxidation lowered the dipole potential of bilayers, although no significant change in N–P vector (from phosphorus to nitrogen in the headgroup) distribution was found. Generally, alterations of membrane properties due to oxidation described by Khandelia and Mouritsen correspond with those found by Wong-ekkabut et al. The main difference between these two studies is the complete reversal of orientation of the anionic chains observed in the oxidized lipids considered by Khandelia and Mouritsen, whereas anionic-terminated lipid chains were not accounted for in the earlier work by Wong-ekkabut et al.

The influence of cholesterol on lateral diffusivity of oxidized lipids was addressed in coarse-grained MD simulations of a DOPC bilayer with 1% of PGPC and up to 50% of cholesterol [49]. Structural properties of the system without cholesterol were in agreement with atomistic-level simulations. In the absence of cholesterol, the lateral diffusion of PGPC was 1.4-fold faster than that of DOPC. This effect was rationalised by a relatively weak incorporation of PGPC in the DOPC membrane. The presence of cholesterol facilitated the incorporation of PGPC in the DOPC matrix diminishing the differences between the lateral diffusion of PGPC and DOPC at 40% cholesterol content, in accord with FCS experimental findings.

The mechanism of membrane damage due to lipid oxidation was studied by two of the present authors for massively oxidized bilayers [13]. Namely, a 100% oxidation of a DOPC membrane was modelled by having DOPC molecules exchanged with oxidation products. Four pairs of DOPC oxidation products, with either both or solely the sn-1 acyl chain terminated with aldehyde were considered (ox1-DOPC and ox2-DOPC). Additionally, the presence of short-chain products (either aldehydes or hydrocarbon) was accounted for. In the systems with both acyl chains oxidized the membrane was unstable due to the presence of large water defects which lead to significant water permeability across the bilayer. As a result, the bilayer structure was destroyed in ~10 ns which led to the formation of micelle-like structures. In the case of systems with only one acyl chain oxidized, the bilayer structure was not destroyed in the simulation timescale (up to 100 ns). Reorientation of oxidized chains toward the water phase was observed; however, not all chains were reoriented since they were terminated by uncharged aldehyde groups, which is in accord with the previous computational results for aldehyde-terminated oxidized lipids [45,47]. Short-chain products were incorporated in the stable bilayers. In the stable systems, membrane thickness decreased in the membrane containing the aldehyde short-chain product and increased in the system with the hydrocarbon short-chain product, the latter due to the hydrophobic character of the oxidation product molecules which resided predominantly near the bilayer centre and thus prevented membrane shrinkage. Thus, the thickness of the oxidized bilayer was shown to be dependent on the type of short-chain products, which goes beyond the conclusions of earlier studies where short-chain products were omitted and solely the reduction of membrane thickness was observed upon oxidation [45,47]. APL in the stable systems increased by up to 32%, mainly due to the enhanced formation of water defects in the bilayer. Increased water permeability with respect to the non-oxidized bilayer was observed in all membranes considered. Moreover, transient transmembrane water pores were formed in the stable oxidized bilayers, with pore opening and closing observed in tens of nanoseconds timescale. This is in accord with both the computational [45] and experimental [62] results regarding an increase of permeability in oxidized lipid membranes. Destruction of membrane upon oxidation was shown to be caused by the formation of water defects and increased water permeability,

which is in accord with a postulate given by Wong-ekkabud and co-workers [45].

In summary, at the molecular level, lipid oxidation induces numerous changes structural properties of membranes. Oxidized chains of OXPCs bend toward the water phase with the extent of the bending being dependent on the hydrophilicity of the polar groups in oxidized chains. The APL increases while the bilayer thickness is typically reduced. Oxidation may eventually lead to destruction of the bilayer structure. Lateral diffusion of truncated oxidized lipids is faster than that of non-oxidized ones; however, the presence of cholesterol reduces this difference.

3.3.2. Hydration and water permeability

Increased hydration of oxidized membranes was further studied in the joined experimental and theoretical study by Beranova and co-workers [54]. POPC membrane with either 10% of PGPC (anionic) or POVPC (neutral) was studied. Reorientation of oxidized *sn*-2 chains was observed, being almost complete for anionic chains of PGPC (see Fig. 6). APL of POPC + POVPC membrane was close to that of pure-POPC, whereas a decrease of APL was noted for POPC + PGPC bilayer. In the latter case, the reduction of APL was caused by both reorientation of oxidized chains toward the water phase and the presence of sodium cations. MD results were in accord with the SR experiment regarding the increased lipid mobility, both local and lateral, in oxidized membranes. It was concluded that the reversal of oxidized chains leads to increased water penetration, and thus increased lipid mobility in the region of carbonyl groups as probed in SR experiments. The mobility was higher for POPC + POVPC membrane due to incomplete reorientation of the chains which caused a significant number water defects. The increase of lateral diffusivity was higher for the POPC + PGPC system due to the lack of lateral interaction, which limited lateral movements in the case of POPC + POVPC.

Water permeability of oxidized membranes was directly addressed in the study of Lis and co-workers [68] with computer simulations in combination with scattering stopped flow experimental measurements. Properties of bilayers composed of DOPC with pairs of oxidation products (ox1-DOPC, i.e., a lipid with one oxidized chain terminated by an aldehyde group, and a short-chain aldehyde) were investigated as a function of the oxidation ratio. A monotonous increase of APL with increasing oxidation ratio (from ~3% for 15% oxidation, to ~50% for 90% oxidation) was observed as well as a reduction of bilayer thickness. Both the character and the extent of water permeation were shown to strongly depend on the oxidation ratio. At 15–30% oxidation, only individual water molecules spontaneously permeated across the bilayer. At 55–66% oxidation, water clusters originating from water defects in the headgroup region formed and were transferred across the bilayer. At 66% oxidation transient water chains formed and spread across

the membrane. At 75–90% oxidation, the transmembrane water pores were created in the tens of nanoseconds timescale. For oxidation ratios ≥75% the flux of water across the bilayer was independent of the oxidation ratio but the time needed for pore opening increased with membrane oxidation.

In summary, oxidation facilitates water permeability, as well as enhances formation of water defects and pores. The detailed character of water leakage through the membrane depends on the oxidation ratio.

3.3.3. Lipid flip-flop

Volinsky et al. studied the influence of lipid membrane oxidation on lipid transmembrane transfer (flip-flop) [58]. MD simulations employing the potential of mean-force technique were used to estimate the free-energy barrier for the flip-flop of anionic POPS lipid in oxidized POPC bilayer. Oxidation of the membrane (POPC + 10% PONPC) resulted in an approximately 20% reduction of the free-energy barrier (from 120 to 100 kJ/mol). The role of oxidized lipids was mainly in increasing the hydrophilicity of the membrane interior which facilitates the transfer of the hydrated anionic PS head group across the hydrophobic acyl chain region. The aldehyde groups of PONPC interact with water and thus stabilize the trans-membrane pore formed during PS transfer (see Fig. 8). Both experimental and simulation results thus show that lipid oxidation leads to a sizable acceleration of scrambling of PS between the two leaflets of the PC membrane.

In summary, the free-energy barrier of the anionic POPS lipid flip-flop is reduced in the oxidized bilayer.

4. Summary

The results of the individual EPR, fluorescence and MD studies are already summarised at the end of every subsection in Section 3. An overview of the specific effects of the individual oxPCs on the biophysics of a bilayer is given in Table 1.

5. Future perspectives of oxPL research

The main interest in the study of oxidative lipidomics stems from increasing knowledge of the existence of relationships between pathologies and oxidative stress. To date, oxidative stress is increasingly considered an important, if not fundamental, aspect of many pathologies. Oxidative damage to living cells is being actively studied under the genomics and proteomics profile, that is, at the level of functional biomolecules. Yet, though considered mainly under the structural aspect, the phospholipid bilayer also plays an important functional role [5]. The membrane of a cell (or of an organelle) should be considered as the crossroad of all omics, in which their integration and proper functioning are directed and modulated by phospholipids.
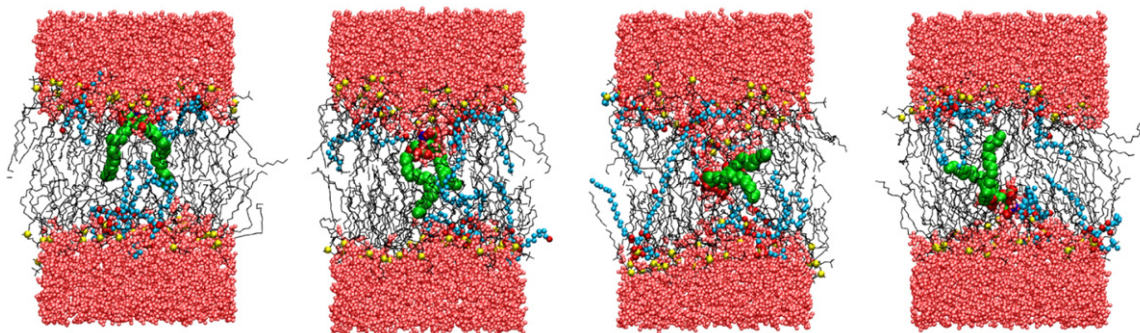
Fig. 8. Four stages of the POPS lipid (green balls) flip-flop (progressing from left to right) across the POPC (black lines) membrane in the presence of PONPC oxidized lipids (blue balls). Water is presented as red balls. Water defects in the vicinity of POPS head group are typically present in the initial stages of the flip-flop process while the transient transmembrane pore is formed as the POPS head group penetrates the hydrocarbon region.
Based on MD trajectories from [58].

Table 1
Biophysical effects of selected oxPCs on model lipid membranes, as studied using EPR, various fluorescence techniques and MD simulations.

oxPC			Effect on biophysical properties of model lipid membrane
Class A	Hydroxy-PCs	HOSAPC	• Pure, SPB: loss of 3-DC EPR anisotropy [50]
		HOPLPC	• Pure, SPB: loss of 3-DC EPR anisotropy [50]
			• Full miscibility with PLPC (similar polarity profiles); 25 mol% in DPPC MLV: T_m lowering (less pronounced than for 25 mol% of PLPC), phase separation induced [14]
			• Pure and in PLPC mixtures: at 40 mol% of cholesterol strong reordering of 3-DC [51]
			• 2–5 mol% in PLPC SPB: orientational order decreased, reorientational dynamics not affected [17]
	Hydroperoxy-PCs	HPSAPC	• Pure, SPB: loss of 5-DSPC [18] and 3-DC [50] EPR anisotropy
		HPPLPC	• Pure, SPB: loss of 5-DSPC [18] and 3-DC [50] EPR anisotropy
			• Fully miscible with PLPC (similar polarity profiles); 25 mol% in DPPC MLV: T_m lowering (less pronounced than for 25 mol% of PLPC), phase separation induced [14]
			• 2–5 mol% in PLPC SPB: orientational order decreased, reorientational dynamics not affected [17]
			• (13-tc − trans-11,cis-9 isomer of HPPLPC) 2.8–50 mol% in PLPC in silico: sn-2 chain reversal (more pronounced than for aldehydes: PONPC and 12-al) and H-bonding with water and lipid headgroups, increased APL, bilayer thinning, order parameter decreased, water defects promoted — increased water permeability [45]
		9-tc	• 2.8–50 mol% in PLPC in silico: sn-2 chain reversal (more pronounced than for aldehydes: PONPC and 12-al) and H-bonding with water and lipid headgroups, increased APL, bilayer thinning, order parameter decreased, water defects promoted — increased water permeability [45]
Class B — truncated PCs	Aldehydes	12-al	• 2.8–50 mol% in PLPC in silico: sn-2 chain reversal (less pronounced than for hydroperoxides: 13-tc and 9-tc) and H-bonding with water and lipid headgroups, increased APL, bilayer thinning, order parameter decreased, water defects promoted — increased water permeability [45]
		PONPC	• Pure and 25 mol% in DPPL: formation of micelles; 25 mol% in PLPC: formation of MLV; in PLPC SPB: heavy loss of EPR anisotropy (5-DSPC and 3-DC) [50]
			• 10, 20, 40 mol% in DPPC air–water monolayers: monolayer expansion, disappearance of LE–LC phase coexistence region (both effects weaker than those of PONPC), LC domains shifted to higher pressures, surface potential lowered, at higher lateral pressures PONPC lost into the water subphase, hypothesis of sn-2 chain reversal up to the point of complete chain extension (so-called extended conformation) [52]
			• Pure, micelles: no binding to cytochrom c (weak association observed) [53]
			• 10 and 16 mol% in POPC LUV: strongly altered polarity and mobility profiles, POPS flip-flop accelerated considerably (even more pronounced for PAzPC); 20 mol% in POPC in silico: stabilization of transmembrane water pores during PS flip-flop, which locally increases the polarity of the bilayer interior [58]
			• 2.8–50 mol% in PLPC in silico: sn-2 chain reversal (less pronounced than for hydroperoxides: 13-tc and 9-tc) and H-bonding with water and lipid headgroups, increased APL, bilayer thinning, order parameter decreased, water defects promoted — increased water permeability [45]
			• 1–25 mol% in POPC in silico: sn-2 chain partial reversal, increased APL, bilayer thinning, order parameter decreased [47]
		POVPC	• Pure and 25 mol% in DPPL: formation of micelles; 25 mol% in PLPC: formation of MLV; in PLPC SPB: heavy loss of EPR anisotropy (5-DSPC and 3-DC) [50]
			• 10 mol% in POPC LUV: increased hydration and mobility of the bilayer carbonyl region; 10 mol% in POPC SPB: slightly increased lateral lipid diffusivity; 10 mol% in POPC in silico: sn-2 partial reversal (preferentially oriented parallel to the bilayer surface), APL not affected [54]
		ox1-DOPC*	• Pure in silico: looping back of oxidized chains toward water phase, large water defects including transmembrane water pores, APL increased, thickness depends on the hydrophobicity of short-chain oxidation products present in the bilayer (decreases for hydrophilic products, increases for hydrophobic ones) [13]
			• 15–100 mol% in DOPC in silico: APL increased, bilayer thinning, water permeation increased: <75 mol% — water transferred as single molecules or small clusters, ≥75 mol% — water pores formed, pore opening depends on oxidation ratio [68]
		ox2-DOPC*	• Pure in silico: bilayer unstable, strong water penetration, bilayer disintegration at nanosecond timescale [13]
	Carboxylic acids	PAzPC	• Pure: formation of micelles [14]; in PLPC SPB: heavy loss of 5-DSPC [18] and 3-DC [50] EPR anisotropy
			• 10, 20, 40 mol% in DPPC air–water monolayers: monolayer expansion, disappearance of LE–LC phase coexistence region, (both effects stronger than those of PONPC), LC domains shifted to higher pressures, surface potential lowered, at higher lateral pressures PAzPC lost into the water subphase, hypothesis of sn-2 chain reversal up to the point of complete chain extension (so-called extended conformation) [52]
			• Pure, micelles: binding to cytochrom c [53]
			• 10 and 14 mol% in POPC LUV: strongly altered polarity and mobility profiles, POPS flip-flop accelerated considerably (more pronounced than for PONPC) [58]
			• 1–25 mol% in POPC in silico: sn-2 chain reversal, decreased APL (due to the presence of sodium cations), bilayer thinning, order parameter decreased [47]
		PGPC	• Pure: formation of micelles; 25 mol% in DPPC: partial formation of micelles; 50 mol% in PLPC: no micelles detected [14]
			• In PLPC SPB: heavy loss of 5-DSPC [18] and 3-DC [50] EPR anisotropy
			• Recovery of bilayer structure upon addition of cholesterol [49]
			• Cholesterol prevents micelle formation at higher PGPC amount in PLPC [51]
			• 1 mol% PGPC in DOPC: and 0–50% cholesterol, reorientation of sn-2 chains towards water phase, headgroups of PGPC shifted toward the water phase and its lateral diffusion faster than DOPC, these two later effects diminished in the presence of cholesterol [49]
			• 10 mol% in POPC LUV: increased hydration and mobility of the bilayer carbonyl region; 10 mol% in POPC SPB: slightly increased lateral lipid diffusivity; 10 mol% in POPC in silico: sn-2 reversal, APL decreased (due to pre presence of sodium cations) [54]

* Studied in silico products of the truncation of DOPC molecule with oxidized chains at sn-1 positions, while the majority of naturally occurring PCs have saturated sn-1 chains and are thus not oxidized in contrast to the polyunsaturated chains occurring preferably at the sn-2 position.

Oxidative modification of these molecules is strongly suspected to impair their role, suggesting that the study and the characterization of oxPL and of lipoperoxidized membranes may bring new clues on how oxidative stress is involved in many diseases. Inasmuch as the study of oxidative lipidomics is expected to shed light on pathology, the development of these studies can be considered worthy.

In our opinion, this development should follow three main routes: mass spectrometry lipidomics, oxidized membrane biophysics, and oxidized (phospho)lipids metabolism.

In fact, owing to the overwhelming amount of oxPL species, their chemical and structural characterization is the indispensable basis for oxidative lipidomics studies. ESI-MS appears to be an invaluable tool to this task, as shown in many instances [69], useful also to build a data bank of standard MS spectra of oxPL for their nanoscale detection in pathologic lipidomes.

Biophysics of membranes containing oxPL species which is in the focus of this review is expected to define how they affect the structure, the properties and functioning of a biomembrane in which oxPL grow after the ROS attack. In addition to pure lipid membrane models, reconstituted models encompassing also functional proteins are devised in order to study how oxPLs affect the lipid–protein relationship, from the point of view of not only enzyme functioning but also of protein folding and functional incorporation into the bilayer. Such studies may help in establishing the extent of oxPL involvement in diseases at the molecular level in the membrane.

An impulse to metabolic studies of oxPL and of oxidized fatty acids should result in more information on how these molecules are related to the rest of the living cell functioning. In fact, deacylation–reacylation of oxPL should yield the basis for oxidative stress repair, as well as catabolism of oxidized fatty acids. A naturally oxidized membrane should be observable through a time window in between the antioxidant defence mechanisms (SOD, glutathione reductase and peroxidase, and the other anti-ROS enzymes) and repair systems. When oxidative diseases are considered, knowledge of these mechanisms would help to understand how much oxPLs contribute to the overall evolution of an oxidatively diseased cell in pathologic organs.

Abbreviations and symbols

12-al 1-stearoyl-2-(12-oxo-*cis*-9-dodecenoyl)-*sn*-glycero-3-phosphocholine
13-tc 1-palmitoyl-2-(13-hydroperoxy-trans-11,*cis*-9-octadecadienoyl)-*sn*-glycero-3-phosphocholine
16-DSPC 1-palmitoyl-2-stearoyl-(16-doxyl)-*sn*-glycero-3-phosphocholine
3-DC 3-doxyl-5-cholestane
5-DSPC 1-palmitoyl-2-stearoyl-(5-doxyl)-*sn*-glycero-3-phosphocholine
9-tc 1-palmitoyl-2-(9-hydroperoxy-trans-10,*cis*-12-octadecadienoyl)-*sn*-glycero-3-phosphocholine
A-ESR angle-resolved electron spin resonance
AFD angle-resolved fluorescence depolarization
BHT butylated hydroxytoluene
Bodipy C$_{12}$-HPC 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine
DHPE-Bodipy N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (triethylammonium salt)
DLPC 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine
DOPC 1,2-dioleoyl-*sn*-glycero-3-phosphocholine
DPH 1,6-diphenyl-1,3,5-hexatriene
DPPC 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine
FCS fluorescence correlation spectroscopy
FRET Förster resonance energy transfer
GUV giant unilamellar vesicles
HOPLPC 1-palmitoyl-2-(13-hydroxy-9,11-octadecanedienoyl)-*sn*-glycero-3-phosphocholine
HOSAPC 1-stearoyl-2-(15-hydroxy-5,8,11,13-eicosatetraenoyl)-*sn*-glycero-3-phosphocholine
HPPLPC 1-palmitoyl-2-(13-hydroperoxy-9,11-octadecanedienoyl)-*sn*-glycero-3-phosphocholine
HPSAPC 1-stearoyl-2-(15-hydroperoxy-5,8,11,13-eicosatetraenoyl)-*sn*-glycero-3-phosphocholine
MLV multilamellar vesicles
NBD nitrobenzoxadiazole
NBD-PC 1-palmitoyl-2-(N-4-nitrobenz-2-oxa-1,3-diazol) aminocaproyl-*sn*-glycero-3-phosphocholine
NBD-PS 1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl) amino]hexanoyl}-*sn*-glycero-3-phospho-L-serine (ammonium salt)
n-DSPC n-doxylstearoyl-phosphatidylcholines
ox1-DOPC 1-(9-oxononanoyl)-2-palmitoyl-*sn*-glycero-3-phosphocholine
ox2-DOPC 1,2-di-9-oxononanoyl-*sn*-glycero-3-phosphocholine
oxPC oxidized phosphatidylcholines
oxPL oxidized phospholipids
PAzPC 1-palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine
PC phosphatidylcholine
PGPC 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine
PGPE-Alexa647 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phospho-N-Alexa647-ethanolamine
PLPC 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine
POVPC 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphocholine
PONPC 1-palmitoyl-2-(9-oxononanoyl)-*sn*-glycero-3-phosphocholine
PPDPC 1-palmitoyl-2-[10-(pyren-1-yl)decanoyl]-*sn*-glycero-3-phosphocholine
PS phosphatidylserine
ROS reactive oxygen species
S structural order parameter
SPB supported planar bilayer
TBARS thiobarbituric acid reactive substances
TDFS time-dependent fluorescence shift method
T_m temperature of the main (gel–liquid crystal) transition

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